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TITLE: Epidemiology of Chronic Wasting Disease: PrPres Detection, Shedding, and Environmental Contamination

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14. ABSTRACT

Chronic wasting disease (CWD) of deer and elk is unique among the transmissible spongiform encephalopathies. Our long-term goal is to better understand the epidemiology of CWD and thus develop strategies for management and control. The specific goals of these studies are to develop sensitive assays for PrPres as a marker for infectivity, and use these techniques to monitor the dynamics and modes of shedding of PrPres from orally infected mule and white-tailed deer and elk. Finally these techniques will be applied to investigating the nature of environmental contamination that may be associated with CWD transmission. Protease resistant prion protein from brains of CWD affected deer and elk (PrPres) and cellular PrPc were purified and used in a variety of detection assays. PrPres was detected using antibody-based techniques, which although substantially more sensitive than any current assay still need improvement. Deer and elk have been and infected orally to determine CWD shedding in vivo. We have now identified several protein biomarkers as indicators of prion infection in urine from deer and elk. As the grant ends we have established a very large bank of various deer and elk tissues and fluids starting prior to infection and periodically throughout the infection.

15. SUBJECT TERMS
Chronic wasting disease, CWD, deer, elk, transmission

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Table of Contents

EXECUTIVE SUMMARY

Obviously the most important goal is to develop an extremely sensitive assay for the infective prion protein. We have made substantial progress since the start of the grant period but are still short of the goal. In a continuation of the proteomics work initiated last year we identified several candidate proteins that are found in urine of infected deer but not in uninfected controls. For some of these we were able to find antibodies and have confirmed the presence of three of these proteins over time using the samples that have been collected for deer over the infection period. Preliminary results indicate that the levels of the proteins increase during the infection period. We have started optimizing the assays on several of the proteins.

As this is a final report I will not reiterate the details presented in the past five years of the grant. However, I will summarize the results and total the publications etc. We are also in the process of filing a patent disclosure on the biomarker proteins we have identified.

INTRODUCTION

Chronic wasting disease (CWD) of deer (*Odocoileus* spp.) and elk (*Cervus elaphus*) is unique among the transmissible spongiform encephalopathies (TSEs) in that it occurs in free-ranging as well as captive wild ruminants and environmental contamination appears to play a significant role in maintenance of the disease. The precise modes of transmission of CWD are not known although we have shown that horizontal transmission and environmental contamination associated with excreta and carcasses may occur (Miller et al., 2004). But maternal transmission does not appear to play a significant role (Miller and Williams, 2003) in maintenance of CWD in cervid populations. Our long-term goal is to better understand the epidemiology of CWD and apply that information to development of strategies for management and control. To that end we are investigating the dynamics and modes of CWD agent shedding from infected mule deer, white-tailed deer, and elk. The approach includes experimentally infecting cervids, serial collections of a variety of biological samples, and assay of these materials by various means to attempt to detect protease resistant prion protein (PrP^{res}). In addition, because of the concern about environmental contamination associated with excreta, we will be collecting and assaying a variety of environmental specimens collected from areas of presumed high, moderate, and low contamination in CWD endemic facilities.

BODY

Aim 1: Develop analytical tools to detect PrP^{CWD} in excreta, blood, and environmental samples.

Biomarker Discovery for Chronic Wasting Disease

Initial Identification of Biomarkers

We have accomplished an extensive analysis of urine from CWD-positive animals. The analysis has identified 11 potential biomarkers, as represented in Table 1. Urine is an ideal source for biomarkers (Aguzzi A, 2004) and we feel strongly that markers found in the urine will also be present in the serum and other tissues of infected animals and our preliminary results are confirming this.

The potential protein markers were identified based on their similarity to known proteins from other mammals, since the deer genome sequence has not been characterized. As such, it is imperative that we accurately identify these proteins. We present them here as CWD-1 thru 11 because we are not completely sure of the proteins identity (except where noted) even though we are seeing antibody cross-reactivity as will be demonstrated in the following pages. Additionally, several of the proteins have a number of isoforms and we are unsure of which isoform we have identified that we are now seeing in blood and urine samples. Research contained within this proposal will appropriately identify the proteins.

Table 1: The identified potential biomarkers of Chronic Wasting Disease.

Biomarker	Possible Physiological Role Summary
CWD-1	Required for a specialized brain endocytosis responsible for generating the synaptic vesicles that store and then release neurotransmitters. Also implicated in Alzheimer's and early loss of cognitive ability.
CWD-2	Reported roles in cell function, clotting, memory and necrosis. Implicated in Alzheimer's and its role in cleaving CWD-1 (see above) and is hypothesized to be partly responsible for early loss of cognitive ability.
CWD-3	Molecular chaperone in the eukaryotic cytosol assisting in protein folding.
CWD-4	A protein truncated in some forms of schizophrenia.
CWD-5	Found in Alexander's disease, a progressive neurological disorder, associated with the destruction of white matter.
CWD-6	Indicator of multi-drug resistance in lung cancer.
CWD-7	A protein scaffold that is involved throughout the cell cycle.
CWD-8	A serine threonine protein kinase involved in mitosis.
CWD-9	Transmembrane protein that plays a critical role in cell adhesion.
CWD-10	Light chain IgG (Serban A, 2004)
CWD-11	An unknown protein that is visibly increased throughout the progression of the disease. Plans for its identification are underway.

Figure 2: **Western blot analysis of 10X concentrated CWD+ deer urine. M= mid infection; L= late infection. LM and LL are the mid and late infection samples from Light Foot, PM and PL are the mid and late infection samples from Pikee while SM and SL are mid and late for Scallop. Neg1-3 represent 3 separate CWD- deer urine samples treated identically to the + samples. The antibody used was commercially available to the rat form of the protein.**

Preliminary screening of samples with biomarker antibodies

Initially to determine which of the proteins have merit as biomarkers for CWD, we purchased commercially available antibodies against the human or mouse forms of the protein, when available. This predisposes the interpretations to be overly cautious. However, the fact that those proteins for which we were able to obtain antibodies are showing up-regulation, or higher expression, in response to the diseased state strongly suggests we are on the right track. We have not tested some of the biomarkers as commercially available antibodies do not exist for them and we

currently do not have funding to generate those antibodies. So we have 6 markers (CWD 1,2,3,4, 10 and 11) that we have positive preliminary data on and that merit further validation.
Given that we developed these protein biomarkers from urine, our initial screens of available proteins focused on urine from both positive and negative animals. **Figure 2** shows the potential that these biomarkers hold. Using an off-the-shelf antibody to another species we obtained positive results. Further, we obtained results that show a tendency of the proteins to be increasingly abundant as the disease progresses. Urine is an ideal source of biomarker material in humans, but may prove less than ideal when trying to test for CWD. However, urine has been identified as an acceptable medium for the development of diagnostic tools (Aguzzi A, 2004).

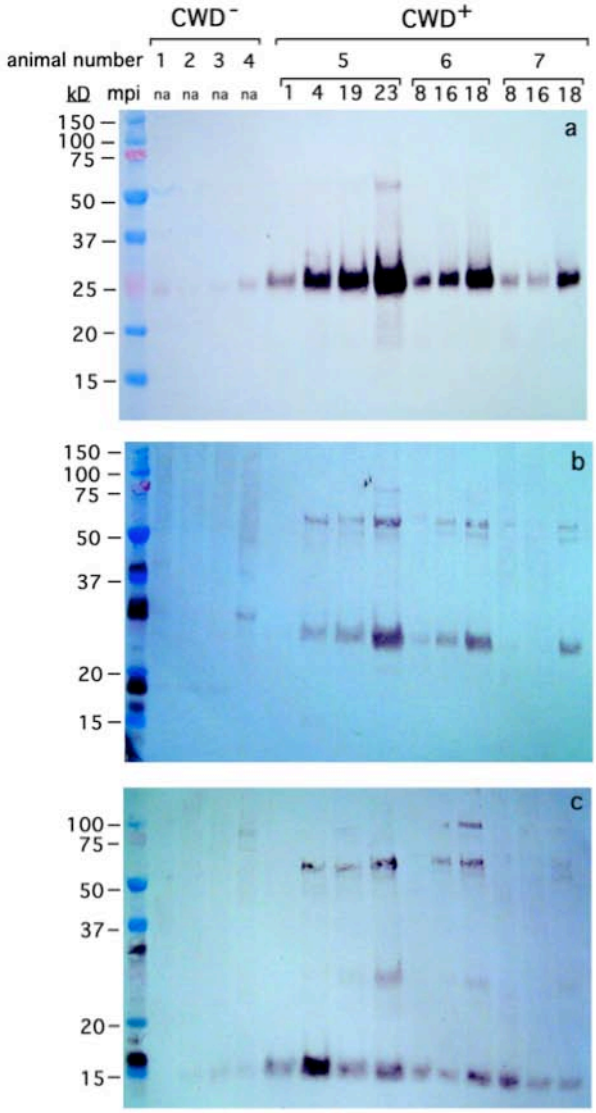

Figure 4. Detection of biomarkers for Chronic Wasting Disease in the urine of White Tail Deer. Proteins in urine collected from four different CWD- and three different CWD+ deer were separated by SDS-PAGE, transferred to nitrocellulose, then probed with antibodies against CWD10 (panel a), CWD2 (panel b), or CWD3 (panel c). Abbreviations: mpi – months post (CWD+) inoculation, na – not applicable. The first lane of each blot has protein molecular weight markers whose sizes (in kilodaltons – kD) are indicated on the left side that blot.

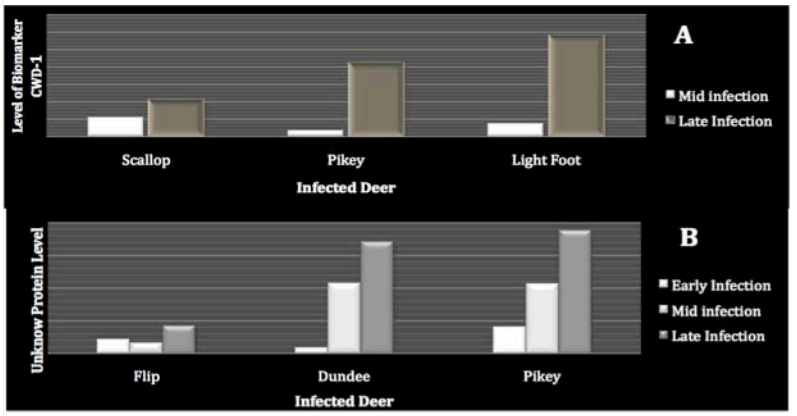

Figure 3
A) Densitometry results for CWD-1 tested in 10X concentrated mule deer urine.
B) Densitometry results for a protein that is visible using a standard ponceau stained membrane that shows an increase in abundance when compared to the other proteins in the urine that appears to follow CWD infection.

Densitometry on the western blot of CWD-1 tested in urine shows a significant trend for the biomarker to be quantifiably higher as the disease progresses (**Figure 3A**).
Figure 3B illustrates that there are still potential biomarkers to be discovered. Although it is beyond the scope of this grant to identify this potential marker, their existence helps to define the potential that protein biomarkers have in diagnosing CWD.
All western blot results shown were performed on 10X concentrated urine. Recent analysis indicates that we can indeed detect the markers in unconcentrated urine (**Figure 4).** Further, the biomarkers can be observed and demonstrate a quantifiable difference throughout the disease state.

Testing in feces was undertaken as another means by which to indicate disease. Some preliminary successes were accomplished (data not shown). However, fecal samples have proved very difficult to analyze. Given that CWD is the only one of the TSE diseases that lends itself to being monitored through feces, we have not chosen to continue this line of research. Serum and urine are far more useful when applied to human TSE's and the TSE's that are known to affect humans, which CWD does not.

Further diversification of the medium of detection to serum broadens the capability of the biomarkers. We have met with limited success in this endeavor largely due to the non specific nature of the antibodies. One clear success on this front is CWD-4 (which according to literature should be represented by a 100kDa and 75kDa band), which is visible at appropriate molecular weights (**Figure 5**) in the infected animal and clearly less prevalent in the non-infected animals.

Figure 5: CWD-4 identified from the urine is available in the serum of infected animals and not in the serum of uninfected animals

We are basing our premise that these will be good biomarkers for the disease on the fact that even with the imperfect antibodies and conditions, we are seeing the protein(s) in the infected animals at well above background levels as the disease progresses in the urine, serum and feces. Perhaps most importantly, we see signal above background very early in the infection.

Preliminary Results Summary

Results obtained thus far are very promising but underscore the need to develop species specific antibodies. The different and complex mediums in which we are testing require specific antibodies if these biomarkers are ever to be used to develop a quick, ante mortem test.

The different TSE diseases lend themselves to detection via biomarkers in different mediums. It is not very efficient to collect urine from wild animals within wild populations such is the case in CWD. As well, blood and serum work well for BSE in the feedlot or slaughter house but urine would seem to be the easiest medium of detection in CJD or vCJD. In both of these mediums we have had success in detecting the markers. The limiting factor is non-specificity to the species. Having multiple biomarkers would allow a testing format that would not rely on a single marker, thus reducing the possibility of getting false positive or negative results. A multiple marker format would also alleviate the argument raised against the use of ESM as an indicator of TSE disease, which is that different individuals have varying levels of transcript (Glock B, 2003) As with all biomarkers there is the potential that the markers may be abundant in other states than the disease of interest. However, a multiple marker format would alleviate that concern. In our proposed system only having one marker indicate positive would not be a positive result. It would require more than one of the markers to indicate the presence of the disease with certainty. Further, our proposed method of utilizing the known light chain IgG (CWD-10) as a fail-safe control alleviates that concern that one marker is insufficient to diagnose the diseased state. With specific antibodies we can determine not only which of the biomarkers are amenable to detection but if they are preferentially detected in one medium and not another.

Given that our laboratory has an extensive library of CWD infected tissues in addition to the facilities and equipment required, we are proposing to develop the biomarkers further using CWD as our TSE of choice. We do expect to be able to test the relevance of our biomarkers in other TSE diseases, but that is beyond the scope of this grant. It is our goal to establish which of the biomarkers, when specific antibodies are used for detection, are useful for confirming the disease. As well, we will establish which biomarkers are useful when applied to urine or serum. With that information we can then develop a test format that will quickly and accurately diagnose the presence of CWD.

KEY RESEARCH ACCOMPLISHMENTS

Determined that high sensitivity detection of the prion protein cannot be accomplished with out sacrificing both false negative and positive results.

Confirmed difficulties reported by others with all of the amplification methods, particularly the false positives, which obviate their standard use for detection.

Identified several proteins that can serve as biomarkers for detection of CWD in live animals from both urine and serum.

Aim 2. Evaluate multiple biological samples collected from experimentally infected mule deer, white-tailed deer, and elk throughout the CWD incubation period.

KEY RESEARCH ACCOMPLISHMENTS

- CWD infections established, confirmed, and monitored to terminus in mule deer and white-tailed deer and elk.
- Serial samples of excreta collected from throughout the disease course from both mule deer and white-tailed deer and elk are available for analysis of prion shedding patterns.
- Genetic influences on disease course in infected white-tailed deer and elk demonstrated, affording opportunities to evaluate the influence of genotype on agent shedding.
- Archived materials shared with other laboratories to advance overall progress on developing sensitive assays for prion detection in blood and excreta, investigating potential routes of prion shedding in deer and elk, and exploring patterns of prion shedding during the disease course.

Aim 3. The goal of this Aim is to determine if PrP^{res} can be detected in samples collected from facilities contaminated with the CWD agent.

KEY RESEARCH ACCOMPLISHMENTS

- CWD infections established and confirmed in mule deer and white-tailed deer.
- PrP^{CWD} demonstrated in tonsil and rectal mucosa biopsies from infected mule deer and white-tailed deer.

- Clinical CWD demonstrated in experimentally infected mule deer and white-tailed deer.
- Archived materials shared with other laboratories to advance overall progress on developing sensitive assays for prion detection in blood.

PUBLICATIONS ARISING FROM GRANT WORK

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(2007) Wolfe, L. L., T. R. Spraker, L. González, M. P. Dagleish, T. M. Sirochman, J. C. Brown, M. Jeffrey, & M. W. Miller. PrP^{CWD} in rectal lymphoid tissue of deer (*Odocoileus* spp.). Journal of General Virology 88: 2078–2082.

(2008) Benjamin D. Brooks, Amy E. Albertson, Justin A. Jones, Jonathan O. Speare, Randolph V. Lewis, Efficient screening of high-signal and low-background antibody pairs in the bio-bar code assay using prion protein as the target, Analytical Biochemistry 382: 60-62.

(2009) Brooks, Benjamin and Lewis, Randolph V. Identification of Problems Developing an Ultrasensitive Immunoassay for the Ante Mortem Detection of the Infectious Isoform of the CWD-Associated Prion Protein, Journal of Immunoassay and Immunochemistry, 30: 135–139.

OTHER COLLABORATIONS ARISING FROM GRANT WORK

Surplus samples collected in the course of investigations supported by this grant have been shared with at least three other collaborating institutions (Rocky Mountain Laboratories, NIH-NIAID; Case Western Reserve University; Institute for Neurodegenerative Diseases, University of California, San Francisco) in the hopes of advancing scientific understanding of CWD in particular and prion diseases in general. Other similar collaborative endeavors will be supported as feasible using materials arising from our work.